

Citrate Uptake in Membrane Vesicles of *Klebsiella aerogenes*

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In whole cells of *Klebsiella aerogenes* grown anaerobically on citrate as sole carbon source, citrate uptake is followed by rapid catabolism of the substrate via the inducible citrate fermentation pathway. Membrane vesicles prepared from such cells take up citrate but do not catabolize it. Vesicles process D-lactate dehydrogenase and the Na⁺-requiring oxalacetate decarboxylase. Citrate is taken up in the presence of Na⁺, and other monovalent cations, such as NH₄⁺, Rb⁺, Cs⁺, or K⁺, do not substitute for Na⁺. Li⁺ appears to act synergistically with Na⁺. Citrate uptake is inhibited by N₂, cyanide, azide, sulfhydryl reagents, dinitrophenol, fluorocitrate, and hydroxycitrate.

Klebsiella aerogenes grows anaerobically on citrate, catabolizing this substrate via the inducible citrate fermentation pathway (2, 3). This pathway consists of two enzymes, citrate lyase (E.C. 4.1.3.6), which is cytoplasmic and catalyses step 1 below, and oxalacetate (OAA) decarboxylase (E.C. 4.1.1.3), a membrane-bound enzyme which catalyses step 2 below.

citrate = oxalacetate + acetate (citrate lyase) (1)

oxalacetate → pyruvate

+ CO₂ (OAA decarboxylase) (2)

OAA decarboxylase is a biotin-containing enzyme and requires Na⁺ for activity; it was originally described by Stern (16).

O'Brien and Stern demonstrated that anaerobic growth of citrate requires sodium in the growth medium (13) and Stern and Sachan (Fed. Proc. 29:932, 1970) showed sodium to be required for the catabolism of citrate in CO₂ in cell suspensions. It is thought that these sodium requirements are due to the sodium requirement of the OAA decarboxylase. Based on the membrane location of the OAA decarboxylase and the observation that citrate is a competitive inhibitor of the enzyme, Sachan and Stern (15) have proposed a model for citrate transport in which OAA decarboxylase serves as a carrier protein for citrate across the cell membrane.

Membrane vesicles have been prepared by the method of Kaback (7), and properties of citrate uptake in the vesicles are described in this paper. Membrane vesicles provide an opportunity for studying the uptake process with-

out appreciable subsequent catabolism as citrate lyase is released during vesicle preparation.

MATERIALS AND METHODS

Culture conditions. *Klebsiella aerogenes* NCTC 418 was used in this study. It was grown anaerobically on citrate medium with the following composition (g/liter): trisodium citrate·H₂O, 20.0; KH₂PO₄, 2.0; (NH₄)₂SO₄, 1.0; MgSO₄·7H₂O, 0.4. The medium was neutralized to pH 7.0 with KOH.

Vesicle preparation. Membrane vesicles were prepared by the method of Kaback (7) utilizing lysozyme and the potassium salt of ethylenediamine tetraacetic acid for the preparation of spheroplasts, with the following modification (Kaback, personal communication); washed cells were suspended to a concentration of approximately 15 g (wet weight) per 500 ml of 30 mM tris(hydroxymethyl)aminomethane-chloride buffer, pH 8.0, containing 0.75 M sucrose and 1 mg of lysozyme per ml. After 5 min with stirring, an equal volume of 5 mM K⁺ ethylenediaminetetraacetic acid (pH 7.0) was added rapidly, and spheroplast formation was allowed to proceed for 1 h at room temperature. Vesicles were routinely purified by using a sucrose density gradient to remove whole cells because it was essential that preparations not contain citrate lyase, a cytoplasmic enzyme released in vesicle formation. Vesicles were frozen in portions of 0.2 ml, either at -55°C (in early preparations) or by immersion in liquid nitrogen (all subsequent preparations).

Uptake assays. The standard incubation mixture consisted of 50 mM potassium phosphate buffer, pH 6.6; 20 mM MgSO₄; 0.48 mM potassium [1,5-¹⁴C]citrate, specific activity 2 to 8 μCi/μmol or 16 μM [1-¹⁴C]proline, specific activity, 261 μCi/μmol; 10 mM NaCl (omitted in the proline uptake assay); 40 mM Li D-lactate; and vesicles (100 to 120 μg of vesicle protein) in a final volume of 50 μliters. In one preparation, the final assay volume was raised to 100 μliters, but reagent and protein concentrations re-

¹ Deceased 15 January 1974.

mained the same. Uptake tubes were incubated at 30 C. The reaction was terminated by the addition of 4 ml of 0.1 M LiCl, followed by rapid filtration on a membrane filter, pore size 0.45 μ m. The filter was washed four times with 4 ml of 0.1 M LiCl, placed in a scintillation vial, and counted in Bray's solution (1) or Beckman Cocktail D in a Nuclear-Chicago Isocap 300 liquid scintillation counter. In each scintillation cocktail, the fluors used were 98% 2,5-diphenyloxazole and 2% *p*-bis-(*o*-methylstyryl)benzene, available as "Omnifluor" from New England Nuclear Corp. Beckman Cocktail D contains 100 g of naphthalene and 8 g of Omnifluor per liter, and dioxane to a final volume of 1 liter. Efficiencies of counting were assessed for each sample by the channels ratio method, and data were expressed as disintegrations per minute, prior to conversion to nanomoles of substrate per milligram of vesicle protein.

Enzyme assays. OAA decarboxylase activity was measured in an assay mixture containing 100 mM potassium phosphate buffer (pH 8.0), 50 mM NaCl, 10 mM OAA, and 100 to 125 μ g of vesicle protein in a final volume of 1 ml. The OAA solution was made up immediately before use, and assay tubes containing OAA were kept at 0 to 5 C at all times except during the incubation and final spectrophotometric readings. The assay tubes were incubated at 30 C for 10 to 20 min. The reaction was terminated by the addition of 0.2 ml of 30% trichloroacetic acid, and the precipitate was removed by centrifugation. The clear supernatant fluid was diluted 1:10 with distilled water, and residual OAA, and subsequently pyruvate, were determined by following the oxidation of nicotinamide adenine dinucleotide, reduced form, at 340 nm with crystalline malate dehydrogenase (E.C. 1.1.1.37) and lactate dehydrogenase (E.C. 1.1.1.27) as described by Stern (16). These assay mixtures contained 100 mM potassium phosphate buffer (pH 6.6), 10 to 100 μ M OAA, 196 μ M nicotinamide adenine dinucleotide, reduced form, 10 μ g of malate dehydrogenase, and 10 μ g of lactate dehydrogenase in a final volume of 1.1 ml. Since malate dehydrogenase was free of lactate dehydrogenase but the latter contained significant traces of the former, it was essential to measure residual OAA first, then pyruvate. Values obtained were corrected for the nonenzymic decarboxylation of OAA during the incubation. Spectrophotometric measurements were made at 23 C with a Zeiss Model PMQ II spectrophotometer. It was found convenient to establish D-lactate oxidation by a similar procedure in an assay mixture containing 20 mM potassium phosphate buffer (pH 6.6), 10 mM MgCl₂, 60 mM NaCl, 40 mM Li D-lactate, and 100 to 110 μ g of vesicle protein in a final volume of 1 ml. After 30 min of incubation at 30 C, 0.2 ml 30% trichloroacetic acid was added, and the precipitate was removed by centrifugation. The undiluted supernatant fluid was assayed for pyruvate with lactate dehydrogenase as described above.

As citrate lyase activity is low in the vesicles, it was possible to estimate this enzyme sequentially with OAA decarboxylase by measuring the Na⁺-dependent release of [¹⁴C]CO₂ from [1,5-¹⁴C]citrate in

a Warburg microreaction flask (7-ml capacity). The assay mixture consisted of 50 mM potassium phosphate buffer, pH 7.0; 5 mM MgSO₄; 50 mM NaCl; 0.3 to 0.5 mM [1,5-¹⁴C]citrate, specific activity, 8 μ Ci/ μ mol; and 100 to 120 μ g of vesicle protein in a final volume of 1.0 ml. The center well contained 0.20 ml of Hyamine, and the sidearm contained 0.20 ml of 30% trichloroacetic acid. After adding the vesicles, the vessel was quickly stoppered and was shaken slowly in a Dubnoff metabolic bath at 30 C. After 10 min, the reaction was stopped by tipping in the acid, and the vessel was shaken for 20 min to insure complete absorption of the liberated ¹⁴CO₂ in the Hyamine. At the end of this time, the Hyamine solution was quantitatively transferred to a scintillation vial and counted in toluene containing 4 g of Omnifluor per liter.

Protein estimation. Protein was measured by the method of Lowry et al. (12) by using bovine serum albumin as standard.

Reagents. Hydroxycitrate isomers and [1,5-¹⁴C]hydroxycitrate, (*pn*_{cit})-(4*S*)-4- configuration, were gifts of O. N. Miller and Ann C. Sullivan of Hoffmann-LaRoche, Nutley, N.J. [2-¹⁴C]fluorocitrate, (*pn*_{cit})-(4*R*)-4- configuration, was synthesized enzymatically by Sachan and Stern from [¹⁴C]malate and fluoroacetyl-CoA (Sachan and Stern, personal communication). Other reagents and enzyme reagents were purchased from commercial sources.

RESULTS

When vesicles were prepared from *Klebsiella aerogenes* grown anaerobically on sodium citrate medium, citrate uptake was observed, and it was dependent on the addition of an electron donor. Uptake in the presence and absence of D-lactate is shown in Fig. 1. The *K_m* for citrate uptake was estimated to be 2.4×10^{-4} M. A

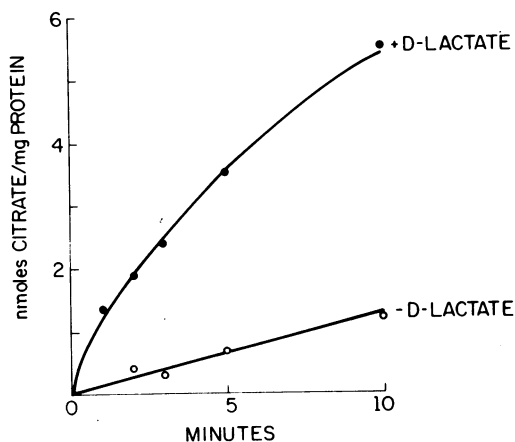


Fig. 1. Uptake of [1,5-¹⁴C]citrate by membrane vesicles of *K. aerogenes*. Citrate specific activity, 3.89 μ Ci/ μ mol.

number of electron donors have been tested, and the ascorbate-phenazine methosulfate system was found to stimulate transport. A summary of some electron donors tested is shown in Table 1. The apparent inhibition of D-lactate energized transport by pyruvate may be due to inhibition of D-lactate oxidation by pyruvate.

In addition to requiring an electron donor, citrate uptake by the membrane vesicles requires sodium. The K_m for sodium was estimated to be 2.5×10^{-3} M. In Table 2, the effects of various monovalent cations on citrate uptake and on proline uptake are shown. It is interesting that sodium, which stimulated citrate uptake, inhibited proline uptake. This observation confirms that of Lombardi and Kaback (11) for proline uptake in vesicles of *Escherichia coli*.

It is also observed that, in the presence of limiting amounts of sodium, there was a lithium synergism effect (Table 3). The effect has been observed for the evolution of carbon dioxide from citrate via the citrate fermentation pathway in cells grown anaerobically on citrate (Sachan and Stern, manuscript in preparation).

The question whether citrate was transported as citrate itself, or is metabolized during the transport step, has been examined by the exper-

TABLE 2. Effects of monovalent cations on citrate and proline uptake in membrane vesicles of *K. aerogenes*

Cation added ^a	Citrate uptake ^b	Proline uptake ^b
None	0.34	0.62
Na ⁺	2.89	0.32
NH ₄ ⁺	0.40	0.55
Rb ⁺	0.34	0.53
Cs ⁺	0.34	0.55

^a For citrate uptake 10 mM, and for proline uptake 20 mM.

^b Nanomoles per milligram of protein per 5 minutes.

TABLE 3. Action of lithium on citrate uptake

Sodium (mM)	Citrate uptake ^a		Stimulation by Li ⁺ (nmol)
	Control	Plus 40 mM Li ⁺	
0	0.23	0.44	0.21
1.0	0.49	0.99	0.50
2.5	1.03	1.59	0.56
10.0	1.67	1.99 ^b	0.32

^a Nanomoles per milligram of protein per 10 minutes.

^b Without potassium D-lactate, 0.29.

TABLE 1. Effects of substrates and cofactors on Citrate uptake in membrane vesicles of *K. aerogenes*

Substrates ^a	Citrate uptake ^b
None	0.31
D-Lactate	1.43
D-Lactate + NAD	1.64
D-Lactate + FAD	1.64
D-Lactate + GSH	1.38
D-Lactate + DTT (1 mM)	1.26
D-Lactate + pyruvate	0.81
L-Lactate + NAD	0.21
Ascorbate + PMS	1.72
Succinate	0.16
Succinate + FAD + DTT (10 mM)	0.22
Fumarate + NADH	0.25
α -Glycerophosphate	0.17

^a Abbreviations: NAD, nicotinamide adenine dinucleotide; FAD, flavin adenine dinucleotide; GSH, glutathione; DTT, dithiothreitol; PMS, phenazine methosulfate; NADH, reduced form, nicotinamide adenine dinucleotide. Concentrations of substrates and cofactors: D-lactate, 40 mM; NAD, 1 mM; FAD, 0.2 mM; GSH, 10 mM; pyruvate, 40 mM; Na⁺ ascorbate, 40 mM; PMS, 0.2 mM; succinate, 40 mM; fumarate, 1 mM; NADH, 1 mM; and α -glycerophosphate, 40 mM.

^b Nanomoles per milligram of vesicle protein per 5 minutes. Concentrations of substrates and cofactors.

iment shown in Fig. 2, in which only unlabeled citrate chased the radioactive label remaining in the vesicles after a 10-min incubation to any significant extent, although both bicarbonate and acetate appear to have stopped subsequent uptake of citrate.

A number of inhibitors prevented citrate uptake in the vesicles, and their effects are summarized in Table 4. All four isomers of hydroxycitrate inhibited transport of citrate; only the (*pn*_{cit})-(4*R*)-4-isomer of fluorcitrate was tested, and it inhibited transport.

Fluorcitrate bound to the vesicles (Table 5), but the amount bound did not increase over 20 min, and the binding was not affected by D-lactate. In contrast, the labeled isomer of hydroxycitrate, (*pn*_{cit})-(4*S*)-4-, was actively taken up by the vesicles (Fig. 3), and the uptake exhibited requirements for Na⁺ and D-lactate. The K_m for the uptake of (*pn*_{cit})-(4*S*)-4-hydroxycitrate was estimated to be 4.5×10^{-4} M. It is not clear why the amount of hydroxycitrate accumulated was higher than the amount of citrate accumulated, but it may be due to some citrate cleavage within the vesicles by residual citrate lyase. The residual citrate lyase activity in the vesicles has been measured at a level of 1 to 3 nmol per mg of vesicle protein per min, an activity which would be sufficient to affect the

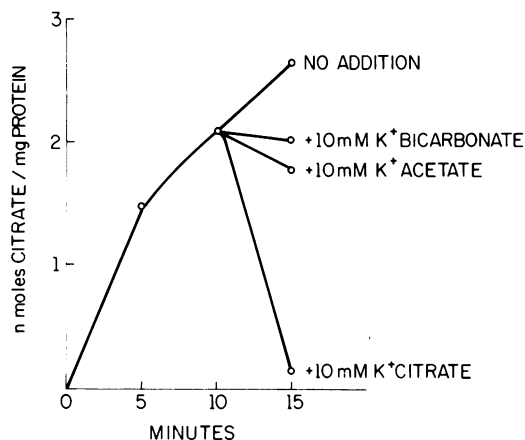


FIG. 2. Chase experiment with $[1,5-^{14}\text{C}]$ citrate. After 10 min of incubation, unlabeled bicarbonate, acetate, or citrate was added. Citrate specific activity, $3.89 \mu\text{Ci}/\mu\text{mol}$. Assayed in a final volume of 100 μliters .

TABLE 4. Inhibitors of citrate uptake

Inhibitor	Concn (mM)	Inhibition (%)
Arsenite	1	10.3
Arsenate	50	2.4
N-ethylmaleimide	4	51.3
PMB ^a	0.1	85.3
Iodoacetamide	4	3.3
Cyanide	10	100
Dinitrophenol	1	100
	0.1	68.8
Oligomycin	0.2	29.7
	0.02	9.2
Oxamate	30	11.9
Azide	10	100
	1	58.2
Fluorocitrate		
(<i>p</i> n _{cit})-(4 <i>R</i>)-4-	3.6	86.7
Hydroxycitrate		
(<i>p</i> n _{cit})-(4 <i>S</i>)-4-	2.5	60.6
(<i>p</i> n _{cit})-(2 <i>R</i>)-2-	2.5	81.7
(<i>p</i> n _{cit})-(4 <i>R</i>)-4-	2.5	72.7
(<i>p</i> n _{cit})-(2 <i>S</i>)-2-	2.5	54.0
Fluorpyruvate	2.5	22.0
cis-Aconitate	10	11.9
trans-Aconitate	10	0
Tricarallylate	10	4.3
D,L-Isocitrate	20	0

^a PMB, *para*-Mercuribenzoate; incubation time, 3 min.

citrate accumulation. (*p*n_{cit})-(4*S*)-4-Hydroxycitrate is an inhibitor of citrate lyase (Sachan and Stern, personal communication), and would not be expected to be cleaved by the enzyme. It should also be pointed out, however, that the results of the chase experiment do not indicate

that a significant portion of the intravesicular $[^{14}\text{C}]$ was present in acetate rather than citrate. Because of the volatility of acetate on chromatography, we have not been able to demonstrate the presence or absence of radioactive acetate directly by chromatographic techniques.

DISCUSSION

Citrate uptake as described in this paper is similar to the uptake of amino acids and sugars found in membrane vesicles by Kaback and co-workers (reviewed in [8]) in being energized by the oxidation of D-lactate or the ascorbate-reduced phenazine methosulfate system. Our cells were grown anaerobically and metabolized citrate by the citrate fermentation pathway; during growth, citrate uptake must be energized by an anaerobic system, and the citrate carrier,

TABLE 5. Fluorocitrate^a binding by vesicles

Time (min)	Vesicle protein (nmol/mg)
1	1.15
2	1.01
5	1.85
10	1.01
20	1.89
5 ^b	1.18

^a $[1-^{14}\text{C}](p\text{n}_{\text{cit}})-(4R)-4$ -fluorocitrate, sp act $0.84 \mu\text{Ci}/\mu\text{mol}$.

^b In the absence of D-lactate.

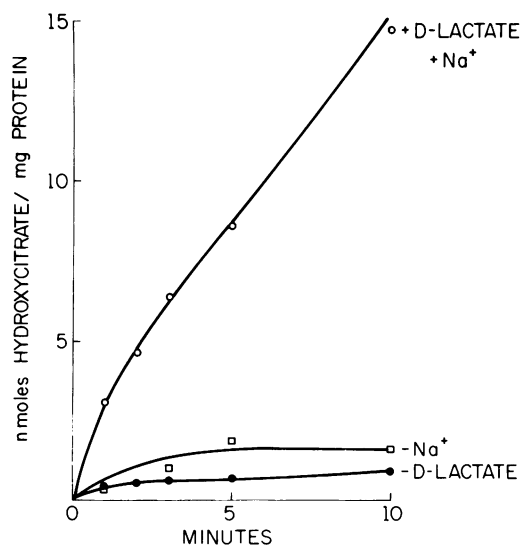


FIG. 3. Uptake of $[1,5-^{14}\text{C}]$ hydroxycitrate, (*p*n_{cit})-(4*S*)-4-isomer, by membrane vesicles of *K. aerogenes*. Hydroxycitrate specific activity, $1.5 \mu\text{Ci}/\mu\text{mol}$.

which may be induced together with the enzymes of the fermentation pathway, must operate with an anaerobically derived source of energy. This energy system remains to be explored.

We have observed citrate uptake energized by the residual electron transport-linked uptake process, which appears not to have been fully repressed in anaerobically grown cells. The levels of citrate taken up are low, but this approach allows us to observe uptake in vesicles without subsequent rapid catabolism of citrate, and parameters of citrate uptake which we observed correlate with similar parameters for uptake and catabolism observed in cell suspensions. Future experiments will examine the mode of anaerobic energization of citrate transport.

The affinity of the vesicles for citrate approximates the natural citrate affinity of the whole cells grown anaerobically. The estimated K_m for vesicular citrate uptake, 2.4×10^{-4} M, was similar to the K_m for the overall process of citrate uptake and catabolism to CO_2 , 1.43×10^{-4} M, in cells grown anaerobically on sodium citrate, as reported by Sachan and Stern (15). This low affinity for citrate resembles that found in *B. subtilis* by Willecke and Pardee (19). The affinity observed for potassium-dependent transport of citrate in aerobically grown *K. aerogenes*, strain UGa-1, by Wilkerson and Eagon (4, 17) is higher, 2.5×10^{-6} M. The possibility that there may be several citrate transport systems, with separate roles in aerobic and anaerobic growth, as well as strain differences in citrate transport clarified in a recent paper by Wilkerson and Eagon (18), seems quite likely.

The requirement for sodium in citrate uptake in the vesicles resembles the growth requirement for sodium when *K. aerogenes* NCTC 418 grows anaerobically on citrate (13). We do not know yet whether the sodium is co-transported with citrate, as magnesium is in the citrate transport system in *B. subtilis*, as shown by Willecke et al. (20). If the model proposed for citrate transport in *K. aerogenes* by Sachan and Stern (15) is correct in that the sodium-requiring OAA decarboxylase also serves as a carrier protein for citrate, it may be that the sodium requirement for citrate transport is quite specific in this system.

We have not observed a high rate of transport of citrate in the vesicles, compared with the rate of OAA decarboxylase in the vesicles and the overall rate of CO_2 production from citrate by the citrate fermentation pathway in cell suspen-

sions. Extrapolation from the curve for hydroxycitrate uptake, which gives the highest observed rate of uptake, allows an estimate for the first minute of $3.2 \text{ nmol per mg of vesicle protein per min}$. The activity of OAA decarboxylase in the vesicles is 0.4 and $0.1 \text{ } \mu\text{mol per mg of vesicle protein per min}$, which would allow a more rapid flux of citrate through the citrate fermentation pathway. Measurement of transport in vesicles prepared to preserve the anaerobic energy-coupling mechanism will hopefully help to reconcile this discrepancy.

One useful feature of the observation of $(pn_{\text{cit}})-(4S)-4\text{-hydroxycitrate}$ uptake is that it suggests that this citrate derivative may be used to study uptake without subsequent catabolism in whole cells possessing the transport system described in this study.

The role of fluorocitrate in bacterial citrate transport is apparently different in different systems. *B. subtilis* has been shown by Oehr and Willecke (14) to transport $(pn_{\text{cit}})-(4S)-4\text{-fluorocitrate}$, but our evidence indicates that fluorocitrate is not transported in vesicles of *K. aerogenes* grown anaerobically on citrate. In *Pseudomonas fluorescens*, Lawford and Williams (10) have shown that fluorocitrate causes intracellular citrate accumulation at the same time that it inhibits CO_2 evolution from citrate. These observations are most easily explained if fluorocitrate is inhibiting aconitase (5), as these authors point out, and such inhibition implies that fluorocitrate has been transported into the cells.

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